Direct ^{31}P NMR spectroscopic measurement of phosphorus forms in dairy manures

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Abstract

Quantitative assessments of the distribution of phosphorus (P) from agricultural sources in environmental matrices require the simultaneous identification of changes over time among the multiple forms of P. Organic P is synthesized from inorganic P by living plants and microbes. These same organic forms can agriculturally revert back to inorganic forms of P as the biomass degrades. In plants consumed by animals, this degradation forms manures. Since about half the P in poultry manures can be organic (phytate P), measuring only inorganic P would account for the environmental fate of about half the P from poultry manures. Since dairy cattle are ruminants that have the digestive phytase enzymes which convert phytate into inorganic P, dairy manures were predicted to contain primarily inorganic P. Instead, phytate was a major component in almost all the agriculturally diverse dairy manures examined. Although digested plant material may have been effectively converted to inorganic P (e.g., absorbed as a nutrient), much of the plant material that remained undigested was not, i.e. it retained the form of P present in the plant material before it was ingested.

Organic P must be strongly held within such a matrix, or the ruminant digestive fluids would have solubilized and hydrolyzed it. Solid state ^{31}P MAS NMR techniques enabled analysis of the forms of P in manure samples from commercial dairy farms without chemically altering the manures. This technique avoids the possibility of hydrolyzing phytate P into organic P via the harsh chemical extraction procedures used to solubilize the organic P. Organic and inorganic P can complex with cations such as Fe^{+3} and Ca^{+2} present in animal diets, but forage is predominantly an organic matrix. Mineral content at the levels in manures, unlike those in soils, did not interfere with the ^{31}P NMR spectroscopy of these intact solids. Organic and inorganic forms of P (including inorganic P complexed with Ca^{+2}) were simultaneously identified in dairy manures by this technique. Organic P (i.e., phytate) was a primary reservoir of P in dairy cattle manures, indicating a significant fraction of plant phytate P in manures can remain as undigested phytate P. The forms of P that build up in manure treated agricultural soils over time depend upon the forms of P that are being added to this reservoir.

Key words: ^{31}P NMR, solids, organic P, phytate, dairy manures, inorganic P, environment, kinetics.

Introduction

Phosphorus (P) is an essential nutrient for both plants and animals, but excess P can enter streams, rivers and groundwater. While biochemical processes convert inorganic P into organic forms, other processes can transform organic P back into inorganic P. Agricultural processes and practices distribute and recycle P among plants, animals, and the environment. The more effectively it is recycled between plant and animal pathways, the more efficiently biological P can be utilized. Soil nutrient P loss is thereby also minimized. Yet unless the forms of P present in soil, plant or animal reservoirs are biochemically compatible, recycling of P between any two of these reservoirs will necessarily follow slow and complicated pathways.

Fertilizer P is typically applied to soil in the inorganic form (as phosphate) or in an organic form (as phytate in manure). The forms of P after incorporation into soil can be organic or inorganic, soluble or insoluble. This soil reservoir matrix is both biochemical and geochemical, yet research has focused primarily on the geochemical portion of this matrix.

Various soil types (differing in soil structure as well as in chemical composition) have different affinities for the inorganic P ions found in soils [1]. Because soils are primarily mineral, the matrix effects, like the formation of insoluble phosphates, can be predominantly mineral. The loss of P has been correlated to the flow of particulates [2] as in soil erosion. By this analysis, P loss would be similar to nitrogen or potassium loss present in the same soil matrix. In a recent hydrological study, spatial variability in P loss and erosion were compared [3]. A weak correlation ($R^2 = 0.6184$) was found between watershed P index (correlated with soluble P) and watershed discharge (soil loss), but the correlation does not take into account that soluble P can be lost even if there is no soil erosion. If movement of soil particulates were the primary mechanism of P loss from soil adding specific metal cations including Fe^{+2}, Ca^{+2} and Al^{+3} to immobilize P in soils [4] would be an ineffective P management strategy.

Determining P loss in manure-treated soils is complicated by the large variability in soil types [5-7]. Soil chemical composition and soil hydrology are both complicating factors in predicting inorganic P fate. Adding organic P to soils makes the process even more complicated. ^{31}P Nuclear Magnetic Resonance (NMR) Spectroscopy enables measurement of organic and inorganic forms of P in soils simultaneously both with and without chemical extraction [8, 9]. Although none was apparently extracted from a set of sandy loam soils [10], the dominant form of organic P extracted from soils was found to be phytate, inositol hexa-phosphate (the

P monoester) [11]. Other published results from soil extracts determined 70-85% of the organic P can be the monoester [12]. Additional organic P components in soils have also been extracted, concentrated and identified [13]. Yet, independent of the chemistry of soil types which can also contain unique microbial environment individual to a soil, and independent of any chemical or morphological heterogeneity in the manure incorporated into a soil, adequate interpretation of analytical results is dependent on knowing the forms of P that had initially been added to the soil.

Plants and microbes synthesize organic P from inorganic P. Crops, including maize, concentrate P primarily as phytate in their seeds [14]. Phytate P is also absorbed by plants as a metal complex [15]. Naturally occurring phytase enzymes in soils, especially from fungi, gradually degrade phytate back into its inorganic form [16].

Plants that contain P primarily as phytate are routinely consumed by farm animals. However, poultry and swine require inorganic P dietary supplements, because non-ruminants cannot release the nutrient inorganic P from phytate. ^{31}P NMR was used to trace the forms of P in poultry, from plant to animal feed to manure [17]. Almost all P in maize was found to be phytate; the same maize was formulated with inorganic P into several poultry meal diets; the individual diets were fed to individual poultry and the corresponding poultry manure was collected; each component was analyzed for forms of P. Half the P in the poultry diet was phytate and half was inorganic P and similarly half the P in manure was phytate. Poultry manure has also been incorporated into numerous types of soil [18], but again inorganic P accounted for only about half of the P added to soils.

^{31}P NMR techniques have been used to identify P composition in swine manures [19]. Studies of composite sludges have determined forms of P [20-22], but studies on the fate and forms of P in swine manures that do not control for swine diet composition may not produce reproducible, self-consistent results. Changes between the forms of P in heterogeneous soils are difficult to identify when swine manure applied to soils itself is comparably heterogeneous in P composition.

Dairy cattle do not require nutrient P supplements in their diets [23]. As ruminants, their digestive systems contain the enzyme phytase to release inorganic P from phytate. Assuming the phytate hydrolysis routinely goes to completion, P composition in bovine manure would be independent of the composition of bovine diet. Although the organic component of P was not chemically analyzed in the study, the inorganic and total P fractions were found to vary with the dairy diet [24]. The organic P component in dairy manure could likewise vary with bovine diet.

Dairy manure can be highly variable both spatially and temporally [25]. Incorporating such non-uniform manures into soils adds another major complicating factor in determining the forms and levels of P especially if manure is combined with urine as a waste management technique [26]. Chemical complexation and microbiological mineralization of P can occur both within dairy manures and within soils containing dairy manure. More certain answers about the fate of P in agricultural soils should be accessible from first documenting the primary forms of P which, using existing good farm management practices, are present before incorporation into soil. Spectra were collected to determine the distribution of forms of P present among a large diversity of intact dairy manures. Results were also compared and contrasted with standard chemical tests for P on the same samples. The objective of this study was to use solid state ^{31}P NMR spectroscopy to determine the forms of P in intact dairy manures which are at present being added to agricultural soils.

Methods

Chemical analysis: Chemical analyses were performed on solid dairy manure samples collected from operational dairy farms in Northeastern United States. Samples were selected to include the normal range and diversity of diets and dairy management techniques typically used in the US. The samples were ground to pass through a #20 mesh sieve after drying at 60°C and were stored at –20°C until analysis. The chemical tests for soluble P forms were water-extractable P [4], oxalate-extractable P [28], phytase-hydrolysable P (PHP) [29], carbon and ash content determination. Total carbon was determined by dry combustion, and total ash was determined by heating to 550°C for 3 hrs. Results presented here are from the eleven most diverse of the 107 dairy samples collected for chemical analysis. They were selected to reflect the normal chemical diversity of manures rather than one sample being characteristic of a larger group of dairy manures. Samples for ^{31}P NMR analysis were selected to reflect the diversity of both inorganic and organic P species based on which had the highest and the lowest chemical analysis results for soluble P, PHP, total carbon and ash content.

^{31}P NMR analysis: Solid state ^{31}P MAS NMR with cross polarization (CP) and high power decoupling techniques at 161.97 MHz was used to investigate the organic and inorganic forms of phosphorus (P) in intact dairy manure samples. The spectra were recorded using a 400 MHz Bruker DMX spectrometer. The samples were packed in 7 mm zirconia Bruker rotors for magic angle spinning (MAS) at 4-6.5 kHz. A CP contact time of 2 ms was used with a 4 s recycle delay, and 1000-5000 accumulations were collected for each sample. The ^{31}P reference was set to external ammonium dihydrogen phosphate ($NH_4H_2PO_4$) (ADP) at 0.8 ppm with respect to phosphoric acid. The spinning side bands were identified by changing the MAS spinning rate approximately 15% in individual samples. Standard sodium phytate was used in the same manure matrices to identify the organic P (phytate) peaks.

Results and Discussion

The mineral content (ash) averaged 22.3% (range: 9.62-52.1%) and the total carbon (TC) averaged 42.0% (range: 47.45-25.14%) in the dairy manure samples (Table 1). Thus the manures analyzed were approximately 78% non-mineral (i.e., C plus H, N, O and S) but ranged from being half mineral to half carbon.

The samples analyzed by chemical procedures differed considerably both in the concentration and in the forms of P. They varied from two times more water-soluble P than PHP to almost 50 times more PHP than water-soluble P. On average, three times more soluble P was released by PHP than without the enzyme. The highest amounts of P release, however, were recorded after the oxalate treatment.

Interestingly, the same samples high (or low) in PHP were also high (or low) in the oxalate P test. Oxalate-soluble P results were typically twice the PHP values ($R^2 = 0.87$). Since the PHP test measures P in the manure's organic fraction, and the oxalate-soluble P measures complexation in the inorganic fraction, the concentrations would be expected to be independent of each other.

Table 1. Results of the chemical and physical analysis of dairy manure samples.

Sample #	Water-soluble P (mg kg^{-1})	Phytase hydrolysable P (mg kg^{-1})	Oxalate P (mg kg^{-1})	Total carbon (TC) %	Ash %
33	433	4,560	9,840	43.7	16.3
3	496	9,830	17,000	41.4	23.9
107	763	4,410	9,670	42.2	22.1
5	2,820	3,400	12,100	44.1	23.0
14	3,920	5,220	14,400	45.2	17.4
13	4,400	7,070	18,700	41.2	26.6
8	121	5,590	8,980	45.0	14.8
9	150	4,470	8,430	44.0	20.2
25	278	7,300	12,800	42.4	20.8
35	682	1,340	5,500	25.1	52.1
23	2,190	1,280	5,380	47.4	9.62
Average	1,480	4,950	11,200	42.0	22.4

No corresponding correlation was found between oxalate-soluble P and ash content. The respective samples with the highest and the lowest ash contents had almost the same concentration of oxalate-soluble P. Total ash analysis did not provide component information specific enough to predict oxalate-soluble P concentrations. Carbon analysis also gave no information on the remaining composition of the chemical structure(s) in the manure.

Multiple organic and inorganic forms of P in the selected 11 samples of dairy manures were simultaneously analyzed by solid state ^{31}P MAS NMR. The inorganic and organic P fractions directly associated with paramagnetic ions Fe^{3+} and Mn^{2+} as observed in soils [9] were not observed in the ^{31}P MAS NMR spectra. Paramagnetic metal ions have a high affinity for P [30] and organic P (phytate) anions can complex with multiple metal cations [26, 31] causing interference with the magnetic resonance signals of P [32]. Similar to a peat, which had 50 times less Fe than soils and 200 times less Mn [29], the concentration of paramagnetic species did not significantly interfere with the analysis of P in the dairy manure samples. However, by using a combination of chemical and spectroscopic analysis we have been able to identify the organic and inorganic P species in the manures as explained in the proceeding paragraphs.

All samples showed the presence of phytate. By using cross polarization magic angle spinning NMR experiments (CPMAS), we were able to selectively excite those P nuclei that would receive polarization transfer from ^{1}H nuclei. Figs 1 and 2 show ^{31}P NMR spectra of solid dairy manure samples containing low levels (samples 33, 3, and 107) and high levels (samples 5, 14, and 13) of water-soluble inorganic P respectively (Table 1). The spectra revealed at least two inorganic P (ca 1.5 and 7.7 ppm) and three broad organic P (phytate) (~0.8, 29 and -32 ppm) resonances that are sensitive to cross polarization. The inorganic peaks are evidenced by sharp peaks or sharp shoulders on the broader organic phytate peaks. Previous studies have also shown that the different forms of inorganic P overlap in the ^{31}P NMR spectra of soils and of sludges [9, 21]. Spinning side bands at -30 and 33 ppm often overlapped the broad phytate regions at -32 and 29 ppm respectively at the MAS of 5.5 kHz shown in the figures. The three phytate P resonances in the CPMAS spectra may identify the phytate P sites having an acidic –OH group attached instead of a negatively charged –O⁻ group. Thus, in the manures about half the six P groups on each phytate molecule *in situ* are protonated, consistent with the neutral to slightly acidic pH of the manure. Changing the pH of the manures changes the phytate peak ratios at 0.8, -32 and 29 ppm. This dependence of ^{31}P NMR peak ratios of phytate in manures suggests similar pH dependence could occur in soils and sludges. Thus ^{31}P NMR spectral comparisons among the soluble components of soils or sludges must be approached with caution unless the pH among the samples is similar.

A sharp inorganic P peak at 7.5 ppm dominates the samples in Fig. 1. Most of the inorganic P present in animal manures with soils have shown some Mg-P (Struvite) and Ca-P forms [1, 20]. The P species peaks typically appear in the –2 to 9 ppm region [33, 34]. We assigned the peak at 7.5 ppm to an inorganic Ca-P fraction such as dicalcium phosphate dihydrate which is consistent with its relatively low water solubility (Table 1). The broad peaks at 0.8, 29, and –32 ppm again are due to organic phytate in the sample.

In addition to the inorganic peak at 7.5 ppm, a second inorganic P resonance occurred upfield ca 1.5 ppm (samples 5, 14, and 13) in Fig. 2. This peak partially overlapped the broad phytate peak at a similar chemical shift. Sharp spinning side bands around –30 and 33 ppm indicated that the effective chemical shift anisotropies (CSA) of the ^{31}P resonances were larger compared to the samples in Fig. 1. These samples also had a higher water-soluble inorganic P

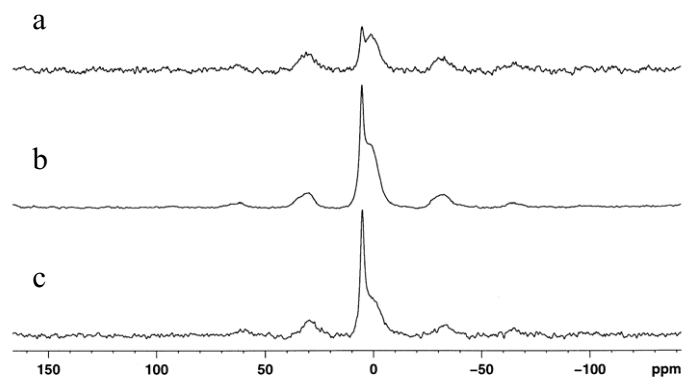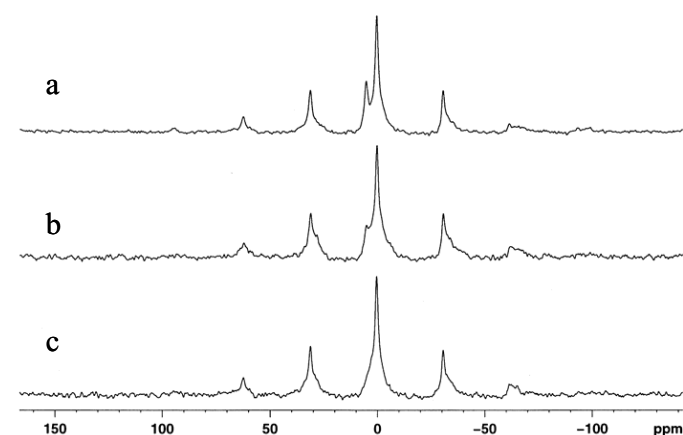**Figure 1.** ^{31}P CPMAS NMR spectra of three dairy manure samples with low levels of inorganic water-soluble P; (a) Sample 33, (b) Sample 3, and (c) Sample 107. Sharp inorganic P peak occurring at 7.5 ppm consistent with its lower solubility is Ca-P. Broad phytate peaks at 0.8, 29, and -32 and two smaller peaks at 59 and -65 ppm increase as phytate concentration increases.

Figure 2. ^{31}P CPMAS NMR spectra of three dairy manure samples with high levels of inorganic water-soluble P: (a) sample 5, (b) Sample 14, and (c) sample 13. Sharp inorganic P peaks occur at 1.5 and 7.5 ppm which indicates two solid forms of inorganic P are present in two samples. Sharp spinning side bands (ssb) occur at 62, 33, -30 and –62 ppm. Broad phytate peaks, overlapped with the sharp inorganic P peaks, occurring at 0.8, 29 and -32 ppm increase with increasing phytate concentration.
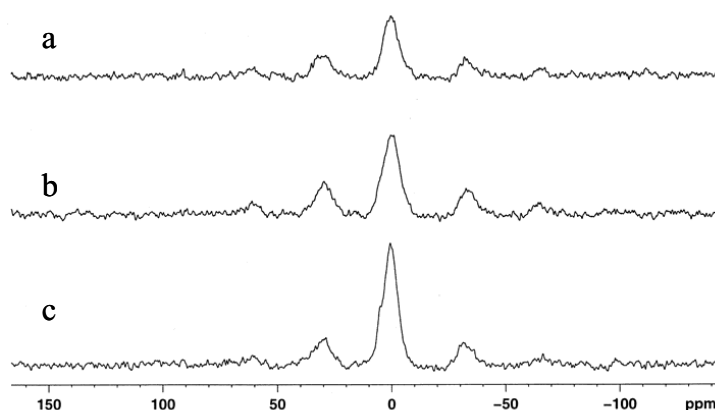
Figure 3. ^{31}P NMR solids spectra of three authentic dairy manure samples containing primarily phytate P: (a) Sample 25, (b) Sample 8, and (c) Sample 9. Broad phytate peaks occurring at 0.8, 29 and -32 ppm increase with increasing phytate concentration. Small additional phytate peaks at 62, -67 and –100 ppm begin to appear above baseline at higher phytate concentrations.

Figure 4. ^{31}P NMR solids spectra of authentic dairy manure samples with very high and very low mineral content respectively. (a) Sample 35 is phytate P plus any inorganic P strongly complexed (broadened) with high levels of minerals in the sample. (b) Sample 23 with broad phytate peaks at 0.8, 29 and -32 ppm and the smaller peaks at 62, -67 and –100 ppm. Standard sodium phytate peak ratios occur at the same chemical shifts when added to the manure samples at the same pH.

phosphate fraction (Table 1). Therefore, the peak at ~1.5 ppm was assigned to a relatively more soluble Ca-P species such as octacalcium hydroxyapatite and/or amorphous calcium phosphate. The phosphate peak at ~ 7.5 ppm is relatively small compared to that of the Ca-P resonance at 1.5 ppm. These samples also indicated total phytate amounts similar to those manure samples in Figure 1.

Fig. 3 shows the ^{31}P CPMAS NMR spectra of manure samples (samples 8, 9, and 25) that contained predominantly organic phytate P with resonances at ~0.8, 29 and –32 ppm. Three smaller phytate P peaks were observed ca ~ 62, -67 and -100 ppm in some of the samples. The different relative intensities of the phytate P resonances in the CPMAS NMR spectra reflects the different chemical environments of the respective phytate P molecular sites and does not reflect the mole fractions of P at the six P sites on the phytate molecule.

The presence of paramagnetic ions such as Fe^{3+} and Mn^{2+} can cause peak broadening of the inorganic P resonances and increase the intensity of their spinning side bands which could overlap the phytate P peaks at the selected MAS. Not recognizing the presence of phytate P can then result in a misinterpretation of the NMR spectra. Dithionate extraction of the paramagnetics as done in previous studies was not carried since these studies have shown that the extraction can also remove certain inorganic P species initially present in samples [9].

The effect of different mineral contents on the ^{31}P NMR spectrum is shown in Fig. 4 (samples 35 and 23). Chemical analysis revealed that sample 35 (Fig. 4a) had a low concentration of total carbon (TC) and a high ash concentration (Table 1). This curve contains phytate P plus any inorganic P strongly complexed (broadened) due to the high levels of minerals in the sample. However, sample 23 (Fig. 4b) shows a high concentration of TC (47%) and a low ash content indicating a low mineral concentration. No sharp peaks corresponding to forms of inorganic P (as in Fig. 2) were observed. In addition, the smaller phytate peaks at 62, –67 and –100 ppm also occur at the same relative peak intensities, and the chemical shifts are consistent with those observed for the standard phytate sample. Adding standard phytate P after first adjusting it to the same pH as the manure sample resulted in the same peaks increasing at the same relative intensity, additional evidence the peaks are not inorganic P broadened due to binding to paramagnetic ions.
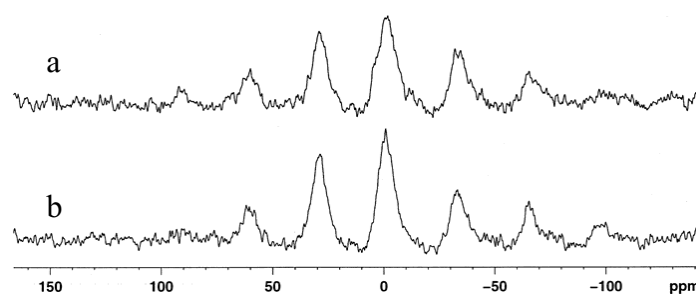
Changing the MAS spinning rate differentiated between the true phytate P peaks and spinning side bands. Peaks that do not change relative positions with a change in spinning rates are not spinning side bands. The relatively low phytate P concentration observed through chemical analysis in sample 23 (1280 mg kg^{-1}) indicates solubilization of phytate P may not be equal in all manures. Compared to the phytate P fractions present in the Fig. 3 samples, the Fig. 4 peaks are broader, consistent with strong complexation of the phytate P sites to anionic and/or cationic components within the organic matrix. The solid state ^{31}P NMR measurement of the intact P forms enabled direct detection of phytate P that was not readily hydrolysable by the chemical assay for phytate P.

Chemical extraction of P followed by ^{31}P solution state NMR, which have been used to characterize extracted inorganic and organic forms of P [35, 36], may not fully solubilize P especially from the high organic fraction components in manures. Moreover, chemical processes that degrade the biochemical matrix in difficult-to-digest manures can also simultaneously hydrolyze phytate P into inorganic P. Poor resolution had been reported in the solid state ^{31}P NMR spectra of composts [37], but similar results were not experienced in manures. The pH and sample heterogeneity could be greater in composts than in manures.

Microbial procedures have been successfully used to release phytate from partially and fully digested swine manures [38], but dairy diets are less digestible than swine diets. Recent work has demonstrated that minerals such as Fe and Al decrease the effectiveness of phytase enzymes [39]. Less effective enzyme activity would again result in an underestimation of phytate P and an overestimation of inorganic insoluble P in poorly digested manures.

Moreover, phytase procedures depend upon both solubilization and reaction kinetics. If solubilization is a slow process relative to its rate of reaction with soluble phytate, the summation of the concentrations of inorganic P released over a relatively short period of time would only correspond to the fraction of phytate which is readily soluble and not to the total amount of phytate present. Once the initial amount of organic P that is present is known, only then can reaction kinetics measurements be important predictors of the environmental fate of P. Mass balance requires knowing the total reservoir of phytate (and not just the amount of readily soluble phytate) because from every phytate molecule in this reservoir, six inorganic P molecules will be released, sooner or later.

Conclusions

The unabsorbed and the undigested solids component in manure can be measured simultaneously and directly using solid state ^{31}P NMR. The environmental consequence of agricultural practices depends upon the forms of P that are present and how long they remain where they are applied. Inaccurate accounting for the fate of organic P (especially phytate P) inherently introduces uncertainty into understanding the process. Whether or not the loss of phytate P has been accurately measured in soils, a reasonable estimate of the annual input of phytate P into agricultural soils should be attainable by measuring phytate P in manures added to soils. An additional source of phytate P is plant material incorporated into soils without having been fed to animals. An accurate measurement of the organic input of forms of P into agricultural soils is a prerequisite to predicting the environmental fate of the inorganic P fraction in soils.

References

[1]Bril, J. and Salomons, W. 1990. Chemical composition of animal manures: a modeling approach. Neth. J. Agr. Sci. **38**:333-351.

[2]Dao, T.H. and Daniel, T.C. 2002. Particulate and dissolved phosphorous chemical separation and phosphorus release from treated dairy manure. J. Environ. Qual. **31**:1388-1398.

[3]Johansson, R.C. and Randall, J.R. 2003. Incorporating economics into the phosphorous index: An application to U.S. watersheds. J. Soil and Water Conserv. **58**:224-231.

[4]Moore, P.A. and Miller, D.M. 1994. Decreasing phosphorous solubility in poultry litter with aluminum, calcium and iron amendments. J. Envir. Qual. **23**:325-330.

[5]Smet, J. de, Hoffman, G., Vanderdeelen, J., van Meirvenne, M. and Baert, L. 1995. Phosphate enrichment in the sandy loam soils of West-Flanders, Belgium. Fertilizer Research **43**:209-215.

[6]Escudey, M., Galindo, G., Foester, J.E., Itilier, S., Page, A.L. and Chang, A. 1997. ^{31}Phosphorous nuclear magnetic resonance in extracts of a phosphorous-enriched volcanic soil in Chile. Comm. Soil Sci. Plant Anal. **28**:727-737.

[7]McDowell, R.W., Condron, L.M., Mahieu, N., Brookes, P.C., Poulton, P.R. and Sharpley, A.N. 2002. Analysis of potentially mobile phosphorus in arable soils using solid state nuclear magnetic resonance. J. Environ. Qual. **31**:450-456.

[8]Bar-Yosef, B., Chang, A.C. and Vega, S. 1993. Organic P transformations, reactions and transport in soils monitored by ^{31}P NMR spectroscopy. Bet Dagan, Israel, BARD.

[9]Lookman, R., Geerts, H., Grobet, P., Merckx, R. and Vlasak, K. 1996. Phosphate speciation in excessively fertilized soil: A ^{31}P and ^{27}Al MAS NMR spectroscopy study. Eur. J. Soil Sci. **47**:125-130.

[10]Pant, H.K., Warman, P.R. and Nowak, J. 1999. Identification of soil organic phosphorus by ^{31}P nuclear magnetic resonance spectroscopy. Commun. Soil Sci. Plant Anal. **30**:757-772.

[11]Rheinheimer, D.S., Anghinoni, I. and Flores, A.F. 2002. Organic and inorganic phosphorus-31 nuclear magnetic resonance in subtropical soils under management systems. Commun. Soil Sci. Plant Anal. **33**:1853-1871.

[12]Zhang, T.Q., Mackenzie, A.F. and Sauriol, F. 1999. Nature of soil organic phosphorus as affected by long-term fertilization under continuous corn (*Zea mays* L.): A ^{31}P NMR study. Soil Sci. **164**:662-670.

[13]Makarov, M.I., Haumaier, L. and Zech, W. 2002. The nature and origins of diester phosphates in soils: a ^{31}P NMR study. Biol. Fertil. Soils. **35**:136-146.

[14]Cileide, C.M.M., Santos, J.C.P., Tsai, S.M. and Vitorello, V.A. 2002. Seed phytate content and phosphorus uptake and distribution in dry bean genotypes. Braz. J. Plant Physiol. **14**:51-58.

[15]Van Steveninck, R.F.M., Van Steveninck, M.E., Fernando, D.R., Horst, W.J. and Marschner, H. 1987. Deposition of zinc phytate in globular bodies in roots of *Deschampsia caespitosa* ecotypes; a detoxification mechanism? J. Plant Physiol. **131**: 247-257.

[16]Wyss, M., Pasamontes, L., Friedlein, A., Rémy, R., Tessier, M., Kronenberger, A., Middendorf, A., Lehmann, M., Schnoebelen, L., Röthlisberger, U., Kusznir, E., Wahl, G., Müller, F., Lahm, H.-W., Vogel, K. and van Loon, A.P.G.M. 1999. Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. Applied and Environmental Microbiology **65**:359-366.

[17]McMurtry, J., Meisinger, J., Schmidt, W.F., Sicora, L., Mitchell, A., Shannon, A. and Sefton, A. 2000. Phosphorous excretion patterns of broilers as affected by dietary phosphorous and phytase. Poult. Sci. **79**: 61.

[18]Crouse, D.A., Sierzputowska-Gracz, H., Mikkelsen, R.L. and Wollum, A.G. 2002. Monitoring phosphorus mineralization from poultry manure using phosphatase assays and phosphorous-31 nuclear magnetic resonance spectroscopy. Comm. Soil Sci. Plant Anal. **33**:1205-1207.

[19]Sands, J.S., Ragland, D., Baxter, C., Joern, B.C., Sauber, T.E. and Adeola, O. 2001. Phosphorous bioavailability, growth performance, and nutrient balance in pigs fed high available phosphorous corn and phytase. J. Anim. Sci. **79**:2134-2142.

[20]Fordham, A.W. and Schwertmann, U. 1977. Composition and reactions of liquid manure (Guelle), with particular reference to phosphate: II. Solid phase components. J. Environ. Qual. **6**:136-140.

[21]Hinedi, Z.R., Chang, A.C. and Lee, R.W.K. 1989. Characterization of phosphorus in sludge extract using phosphorous-31 nuclear magnetic resonance spectroscopy. J. Environ. Qual. **18**:323-329.

[22]Gigliotti, G., Kaiser, K., Guggenberger, G. and Haumaier, L. 2002. Differences in the chemical composition of dissolved organic matter from waste material of different sources. Biol. Fertil. Soils. **36**:321-329.

[23]Board on Agriculture and Natural Resources (BANR) 2001. Nutrient requirements of dairy cattle: seventh revised edition. National Academies Press, Washington, DC, pp. 105-161.

[24]Dou, Z., Knowlton, K.K., Kohn, R.A., Wu, Z., Satter, L.D., Zhang, G., Toth, J.D. and Ferguson, J.D. 2002. Phosphorous characteristics of dairy feces affected by diets. J. Environ. Qual. **31**:2058–65.

[25]Van Horn, H.H., Wilkie, A.C., Powers, W.J. and Norstedt, R.A. 1994. Components of dairy manure management systems. J. Dairy Sci. **77**:2008-2030.

[26]Dao, T.H. 2004. Organic ligand effects on enzymatic dephosphorylation of *myo*-inositol hexa*kis* dihydrogenphosphate in dairy wastewater. J. Environ. Qual. **33**:349-357.

[27]Murphy, J. and Riley, J.P. 1962. A modified single solution method for the determination of phoshate in natural waters. Anal. Chim. Acta. **27**:31-36.

[28]He, Z. and Honeycutt, C.W. 2001. Enzymatic characterization of organic phosphorous in animal manure. J. Environ. Qual. **30**:1685-1692.

[29]Shand, C.A., Cheshire, M.V., Bedrock, C.N., Chapman, P.J, Frazer, A.R. and Chudek, J.A. 1999. Solid-phase ^{31}P NMR spectra of peat and mineral soils, humic acids and soil solution components: influence of iron and manganese. Plant and Soil **214**:153-163.

[30]Bebot-Brigaud, A., Dange, C., Fauconnier, N. and Gerard, C. 1999. ^{31}P NMR potentiometric studies of phytic acid ionization and complexation properties toward Co^{+2}, Ni^{+2}, Zn^{+2} and Cd^{+2}. J. Inorg. Biochem. **75**:71-78.

[31]Gilbin, R., Gomez, E. and Picot, B. 2000. Phosphorous and organic matter in wetland sediments: analysis through gel permeation chromatography (GPC). Agronomie **20**:567-576.

[32]Sutter, B., Taylor, R.E., Hossner, L.R. and Ming, D.W. 2002. Solid state ^{31}phosphorous nuclear magnetic resonance of iron-, manganese-, and copper-containing synthetic hydroxyapatites. Soil

 Sci. Soc. Am. J. **66**:455-463.

[33]Frossard, E., Tekely, P. and Morel, J.L. 1994. Chemical characterization and agronomic effectiveness of phosphorus applied as a polyphosphate-chitosan complex. Fert. Res. **37**:151-158.

[34]Roswell, W.P., Waugh, J.S. and Yesinowski, J.P. 1980. High resolution variable temperature ^{31}P NMR of solid calcium phosphates. J. Am. Chem. Soc. **102**:2637-2643.

[35]McDowell, R.W., Condron, L.M. and Mahieu, N. 2003. Analysis of phosphorus in sequentially extracted grassland soils using solid state NMR. *Communications in Soil Science and Plant Analysis*. **34**:1623-1636.

[36]Turner, B.L. 2004. Optimizing phosphorous characterization in animal manures by solution phosphorous-31 nuclear magnetic resonance spectroscopy. J. Environ. Qual. **33**:752-766.

[37]Cade-Menum, B.J., Berch, S.M., Preston, C.M. and Lavkulich, L.M. 2000. Phosphorous forms and related soil chemistry of Podzolic soils on northern Vancouver Island: A comparison of two forest types. Can. J. For. Res. **30**: 1714-1725.

[38]Klemm, P.A., Lommen, A., De Jonge, L.H., Van der Klis, J.D., Jongbloed, A.W., Mroz, Z. and Beynan, A.C. 1999. Quantitation of inositol phosphates using 31P nuclear magnetic resonance spectroscopy in animal nutrition. J. Ag. Food Chem. **47**:5116-5121.

[39]Heighton Davies, L., Siefert, R.L. and Schmidt, W.F. 2004. Availability of phytic acid complexes of ferric and ferrous iron to enzymatic dephosphorylation by *Aspergillus Ficcum*. Proceedings of American Society of Limnology and Oceanography Savannah, GA. June 13-18.